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DEVELOPMENT AND CHARACTERIZATION OF A RECOMBINANT ORF VIRUS
VECTOR EXPRESSING THE SPIKE PROTEIN OF PORCINE EPIDEMIC
DIARRHEA VIRUS

BY

KYLE HAIN

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Biological Sciences

South Dakota State University

2016

DEVELOPMENT AND CHARACTERIZATION OF A RECOMBINANT ORF VIRUS
VECTOR EXPRESSING THE SPIKE PROTEIN OF PORCINE EPIDEMIC
DIARRHEA VIRUS

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Biological Sciences degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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I'd like to dedicate this to all those who accompanied me on this wild ride; to those who are gone, and those still by my side. I thank you all.

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ABBREVIATIONS

ADRDL	Animal Disease Research and Diagnostic Laboratory
APC	Antigen presenting cell
ASW	Animal Resource Wing
CBP	Chemokine binding protein
COX-2	Cyclooxygenase 2
CPE	Cytopathic effect
CSFV	Classical swine fever virus
DAMPs	Damage associated molecular patterns
DC	Dendritic cell
dsRNA	Double-stranded RNA
E	Envelope
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FFN	Fluorescent focus neutralization assay
GFP	Green fluorescent protein
GM-CSF	Granulocyte/monocyte-colony stimulating factor
GIF	GM-CSF inhibitory factor
IFA	Immunofluorescence assay
Ig	Immunoglobulin
IKK	I κ B kinase
IFN	Interferon
IL	Interleukin
IM	Intramuscular
IMP	Immunomodulatory protein

iNOS	Inducible nitric oxide synthetase
LPS	Lipopolysaccharide
M	Membrane
MEM	Minimal essential media
MOI	Multiplicity of infection
NA	Neutralizing antibodies
NF- κ B	Nuclear factor- κ B
NK	Natural killer
N	Nucleocapsid protein
OD	Optical density
OFTu	Ovine fetal turbinate
ORFV	Orf virus
ORFV-GFP	ORFV-vector expressing GFP
ORFV-PEDV-S	ORFV-vector expressing PEDV-S
OVIFNR	Orf-gene encoding for an IFN resistance factor
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PBS-T	PBS-tween
p.c.	Post-challenge
PDCoV	Porcine-delta coronavirus
PED	Porcine epidemic diarrhea
PEDV	Porcine epidemic diarrhea virus
PEDV-S	Porcine epidemic diarrhea virus spike protein
p.i	Post-immunization
PK	Porcine kidney

PKA	Protein kinase A
PKR	Protein kinase R
PRR	Pattern recognition receptor
PRV	Pseudorabies virus
RHDV	Rabbit hemorrhagic disease virus
RT	Room temperature
rT-PCR	Real-time PCR
S	Spike
sIg	Secretory immunoglobulin
S/P	Sample-to-positive
ST	Swine testicle
TC	Transcutaneous
TCID ₅₀	Tissue culture infectious dose resulting in $\geq 50\%$ cytopathic effect
TGEV	Transmissible gastroenteritis virus
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF- α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
2-5A	2-5 adenylylase synthetase

ABSTRACT

DEVELOPMENT AND CHARACTERIZATION OF A RECOMBINANT ORF VIRUS
VECTOR EXPRESSING THE SPIKE PROTEIN OF PORCINE EPIDEMIC
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KYLE HAIN

2016

Orf virus (ORFV), the type member of the genus *Parapoxvirus* of the family *Poxviridae*, causes orf or cutaneous pustular dermatitis in sheep and goats. ORFV is a ubiquitous virus capable of re-infecting its hosts multiple times over time. ORFV causes a non-systemic, self-limiting disease which is usually restricted to the skin surrounding the virus entry sites. ORFV has evolved several immunomodulatory proteins (IMPs) that evade and/or modulate host immune responses to infection and contribute to virus virulence and disease pathogenesis. Given biological properties and unique immunomodulatory properties, ORFV has gained significant attention in recent years for its potential as a vaccine delivery vector for use in veterinary medicine. Here we explored the potential of ORFV as a vaccine delivery vector for use in swine. The spike (S) protein of porcine epidemic diarrhea virus (PEDV) was used as our model antigen and the immunogenicity and protective efficacy of a recombinant ORFV expressing the full length S protein of PEDV (ORFV-PEDV-S) was assessed in pigs. The ORFV-PEDV-S virus was generated by homologous recombination and the DNA sequences of PEDV S were inserted into the *ORFV121* gene locus, an immunomodulatory protein (IMP) encoded by ORFV that contributes to the virus virulence. The resultant recombinant virus was characterized *in vitro* and its immunogenicity and protective efficacy evaluated *in vivo*. Results of our study

show that intramuscular (IM) immunization of swine with the ORF-PEDV-S recombinant virus elicited robust humoral immune responses and protected animals from clinical disease. Additionally, IM immunization with the ORFV-PEDV-S led to reduced virus shedding in feces. The results of this study provide important information on the feasibility of ORFV as a vaccine delivery vector for use in swine.

Chapter 1: Literature Review

Orf virus (ORFV)

Orf virus (ORFV) is the type member of the genus *Parapoxvirus* of the family *Poxviridae*¹. The ORFV genome consists of double-stranded DNA (dsDNA) molecule of approximately 138 kbp in length². The ORFV genome is organized in a central core that is flanked by two terminal variable genomic regions². The core genomic region contains genes that are conserved across different poxviruses and encodes proteins involved in basic mechanisms of virus replication transcription and morphogenesis³. Whereas the genomic ends contain variable genes encoding for immunomodulatory proteins (IMPs) with putative functions on virus virulence, pathogenesis and host range².

ORFV is an epitheliotropic virus and keratinocytes and epithelial cells in the oral mucosa are the major – if not the only – cell types to support ORFV replication in the host^{4,5}. ORFV replicates in the cytoplasm of infected cells, thus the virus packages its own transcription/replication machinery in the virions⁶.

Poxviruses present a distinct transcription strategy that can be broadly divided in three categories (early, intermediate, and late) based on the time that each gene category is transcribed⁷. Early genes are expressed within minutes to the first hours of infection (20 min to 3-4 h), and encode immunomodulatory proteins (IMPs) that play roles in host-immune evasion and virus virulence² or yet factors that will facilitate the expression of intermediate genes^{8,9}. Intermediate genes are usually transcribed between 3-5 hours post infection and encode for transcription factors that regulate transcription of late poxviral

genes ^{9,10}. The products of intermediate and late genes participate in the replication of the virus genomic DNA, and are directly involved in virion assembly and morphogenesis ⁷.

ORFV infection

ORFV is the causative agent of orf or contagious pustular dermatitis, an acute skin condition affecting sheep and goats ^{11,12,13}. Orf typically presents as scabby lesions affecting the skin around the mouth, lips, nostrils, nares, udder, and teats ¹⁴. ORFV enters through damaged skin and replicates in proliferating keratinocytes in the skin or their counterparts in the oral mucosa surrounding the virus entry sites ¹⁵. Following an incubation period that varies between 3-4 days, the disease starts with erythema and progresses through the stages of papules, pustules, vesicles and scabs ⁴. Orf lesions usually resolve within 4-6 weeks and scabs fall off leaving an area of keratinized epithelium covering the site of virus replication ¹⁶. Infections with ORFV are localized to the site of infection and there is no evidence of systemic dissemination of the virus ⁴. ORFV is a ubiquitous virus and the disease occurs worldwide in areas where sheep and goats are raised. Mortality associated with ORFV infection is usually low; however, morbidity may reach up to 90% in young animals ¹³.

One of the unique properties of ORFV is its ability to re-infect animals multiple times; however, in re-infections the disease is usually milder and resolve sooner (within 2-3 weeks) than in primary infections ¹⁷. Presumably, this is due to immunomodulatory properties of the virus, including, for example, the lack of neutralizing antibodies against ORFV in infected animals ¹⁸.

Immune responses to ORFV

The immune responses to ORFV are short-lived and animals can be repeatedly re-infected throughout their life ^{5,18,19}. Immune responses to ORFV are initiated by keratinocytes upon infection ²⁰. Keratinocytes are the most abundant cell type in the epidermis and function as sentinels of the skin. These cells express several pattern recognition receptors (PRRs) at the cell surface or intracellularly that sense invading pathogens and trigger the initial steps of the immune responses ²¹. The PRRs recognize signatures in invading pathogens named pathogen associated molecular patterns (PAMPs). PAMPs recognize molecules that are unique to pathogens and not present on normal eukaryotic cells ²². One specific PRR, called toll-like receptor 3 (TLR-3) recognizes double-stranded RNA (dsRNA), a common product of viral infections ²³. Recognition of PAMPs by the PRRs of keratinocytes and resident immune cells leads to the secretion of pro-inflammatory and immune-stimulating cytokines ²⁰. Cytokines released in response to ORFV replication include interferon- α (IFN- α), IFN- γ , interleukin beta-1beta (IL-beta1), IL-8, and granulocyte/monocyte-colony stimulating factor (GM-CSF) ^{3,24-27}.

Following ORFV infection, the immune response is characterized by the recruitment of CD4+ T helper cells, Natural Killer (NK) cells, and dendritic cells to the site of infection ^{28,29}. It is common to observe an influx of neutrophils, CD8+ cytotoxic T cells, and B cells that accumulate in areas adjacent to or beneath infected cells ¹⁹. It has also been observed that there is an abundance of $\gamma\delta$ T cells during primary infection ³.

CD4+ T cells are the predominant cell type in both primary and secondary infections and are the most important cells in determining the outcome of infection. CD8+ T cells and B cells have also been observed in both primary and secondary infections,

though the role of these cells on virus clearance remains unknown ^{28,30}. Experimental treatment with cyclosporine A, an immunosuppressive drug that inhibits T-cell development, during an ORFV infection resulted in a significant increase in the severity of the disease that could be attributed to a notable decrease in the production of IL-2 and IFN- γ ³¹. A similar experiment using neutralizing antibodies against CD4+, CD8+, and $\gamma\delta$ T-cells revealed that the number of CD4+ T cells was inversely proportional to the severity of the disease. A similar, yet much less dramatic effect was observed with depleted populations of CD8+ or $\gamma\delta$ T-cells ³⁰.

As previously noted, ORFV can re-infect the same animal multiple times. Secondary infections typically present with a less severe form of the disease and resolve sooner than primary infections ³². Notably, during secondary infections by ORFV a significantly higher number of IFN- γ producing cells are observed. There is also an observed upregulation of TNF- α expression, one of the primary inducers of the nuclear-factor kappaB (NF- κ B) pathway. It has been suggested that IFN- γ plays an important role in the early events leading to clearance of ORFV upon secondary exposure ³³.

A hallmark of ORFV infection is the absence of neutralizing antibodies in naturally infected animals ³⁴. Seroconversion against immunodominant antigens has been observed in sheep; however these antibodies don't seem to play a role in protection ⁴. These findings explain why the transfer of antibody from infected mother via the colostrum does not protect lambs from infection ³⁵. Presumably, the lack of neutralizing antibodies against ORFV is one of the primary reasons for the virus to repeatedly re-infect its host. Additionally, immunomodulatory proteins (IMPs) encoded by ORFV are thought to play a role on immune evasion ³⁶.

Immunomodulation by ORFV

Like other poxviruses, ORFV has evolved several mechanisms to evade and modulate host immune responses to infection⁶. ORFV encodes various genes with putative immunomodulatory and virulence functions that likely inhibit host responses to infection. Several of these IMPs will be discussed below.

One of the best characterized virulence factors is the ORFV orthologue of the mammalian vascular endothelial growth factor (VEGF)³⁷. The ORFV VEGF (*ORFV132*) is a *bone fide* virulence factor because it does not inherently evade or avoid host immune responses but contributes to ORFV virulence in sheep. The ORFV VEGF functions in a similar fashion as its mammalian counterpart, stimulating epidermal keratinocyte proliferation, and thus presumably providing more target cells for virus infection and replication³⁸. Notably, deletion of the VEGF gene from the ORFV genome has shown to markedly attenuate the virus and decrease disease severity in sheep³⁹. It has been suggested that VEGF encoded by ORFV enhances virus replication by both increasing the number of susceptible cells and also inhibiting apoptosis in infected cells^{3,18,39}.

ORFV has also evolved various genes that target host immune responses to infection. One of the first identified ORFV IMPs is the interferon (IFN) resistance factor (OVIFNR)⁴⁰. OVIFNR is an a homolog of the well-characterized vaccinia virus E3L gene³⁶ and it functions by preventing detection of viral double-stranded RNA (dsRNA) in infected cells. OVIFNR exerts its function by binding dsRNA, subsequently inhibiting the activation of cellular protein kinase R (PKR) or the 2'-5'-oligoadenylate synthetase enzyme (OAS1). The PKR works by phosphorylating itself and then the α subunit of translation elongation initiation factor eIF2, successfully halting the production of both

cellular and viral proteins ⁴¹, whereas the OAS1 functions by activating RNase L that degrades viral and cellular RNA, successfully inhibiting translational activity ⁴². Both mechanisms lead to an antiviral state following type-I IFN signaling ³⁶.

Another ORFV gene that inhibits host immune responses is the granulocyte monocyte colony stimulating factor (GM-CSF) and interleukin 2 (IL-2) inhibitor (GIF; *ORFV117*). Although the precise function and activity of the GIF is not completely understood, the protein has been shown to bind to GM-CSF and IL-2 (pro-inflammatory cytokine), presumably inhibiting their action ⁴³. GM-CSF plays an important role in the recruitment and production of white blood cells, mainly granulocytes and monocytes ⁴⁴. These cells consist mostly of neutrophils, macrophages, and dendritic cells that play important roles orchestrating immune responses ⁴⁵. IL-2 is a pro-inflammatory cytokine that functions in the development and activation of T cells and NK cells ⁴⁶. Thus, IL-2 is directly involved in the production of IFN- γ , which has been shown to be detrimental to ORFV infection ³¹. Notably, the GIF is secreted from ORFV infected cells and local inhibition of GM-CSF and IL-2 may be advantageous for ORFV replication in keratinocytes.

ORFV also encodes a homologue of mammalian IL-10 ^{47–49}. IL-10 is an immunoregulatory and anti-inflammatory cytokine often functioning by downregulating inflammatory responses, inhibiting proliferation of immune cells and promoting healing ⁵⁰. The ORFV IL-10 (vIL-10) functions in a similar fashion to its ovine counterpart, with the secreted product inhibiting TNF- α and IL-8 production by macrophages and keratinocytes ⁵¹. In addition vIL-10 has been shown to inhibit the production of IFN- γ ⁴⁹.

NF-κB and ORFV

The NF-κB pathway is an important early innate immune mediator involved in orchestrating inflammation and immune responses upon pathogen recognition ⁵². Early signaling by NF-κB and consequent gene transcription play important roles in different areas of innate and adaptive immunity. Given the central role played by NF-κB on modulation of innate and inflammatory responses to infection, it is not surprising that several viruses have evolved mechanisms that specifically interfere with NF-κB activation ⁵³. Notably, ORFV has been shown to encode at least three proteins that target the NF-κB signaling pathway ^{54–56}.

The NF-κB family of transcription factors comprises five proteins (RelA or p65, RelB, c-Rel, p50 and p52) that form homo- or heterodimers and regulate transcription of several genes involved in innate immune responses, cell cycle and apoptosis ⁵⁷. During homeostasis NF-κB members remain in the cell cytoplasm bound to nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) ⁵². IκBα serves as an inhibitory protein that masks the nuclear localization signals of the NF-κB p65-p50 complex, sequestering it to the cytoplasm and preventing its transcriptional activity ⁵⁷.

Activation of the NF-κB pathway can result from a vast array of stimuli including cytokines, and bacterial or viral products ⁵⁸. These stimuli are typically triggered in response to infection, inflammation, or cellular stress ⁵⁹. These signals are recognized by cellular PRRs, resulting in pathway activation. Once activated, NF-κB regulates expression of several genes that function during inflammation, apoptosis, and innate and adaptive immune responses ⁵². Given the role of NF-κB in such critical cellular processes, it is not surprising that many viruses have evolved strategies to modulate activation of this pathway.

ORFV for example encodes at least three genes *ORFV002*, *ORFV024*, and *ORFV121* that have been recently shown to inhibit activation of the NF- κ B signaling pathway.

These proteins target both nuclear and/or cytoplasmic events leading to NF- κ B mediated gene transcription ^{54–56}. The protein encoded by ORFV002 functions by targeting nuclear events that regulate NF- κ B transcriptional activity, suppressing the acetylation of nuclear NF- κ B p65 by p300 ⁵⁶. ORFV002 was found to directly interact with nuclear NF- κ B p65, interfering with the formation of the p300-p65 complex necessary for NF- κ B p65 acetylation. ORFV024 functions in the cytoplasm by inhibiting phosphorylation of IKK alpha and beta ⁵⁴, whereas ORFV121 binds to NF- κ B p65 in the cell cytoplasm inhibiting its phosphorylation and translocation to the nucleus ⁵⁵. Notably, deletion of ORFV121 from the ORFV genome resulted in a markedly attenuated disease phenotype in sheep ⁵⁵. These immunomodulatory properties of ORFV make the virus a promising candidate for vaccine delivery in animal species, with genes encoding IMPs representing promising sites for insertion foreign DNA in ORFV-based vectors.

Use of ORFV as a vaccine delivery vector

Large DNA viruses have long been used as vaccine vectors for delivery of heterologous antigens in human and veterinary medicine ⁶⁰. There are several properties that make viruses attractive candidates as vaccine delivery platforms. Viruses are capable of delivering and expressing heterologous viral proteins directly in target host cells ^{61–63}. Live virus-vectors are also promising in that they can induce humoral and cellular immune response, thus resembling the immunity following a natural virus infection ^{62,64}. The restricted host-range of many large DNA viruses, especially poxviruses, makes them useful in delivering target antigens in non-permissive species ¹⁵. Another advantage of using

poxviral-vectors is that they replicate in the cytoplasm ⁶⁵, which eliminates the possibility that the viral DNA will be incorporated in the host genome ⁶⁶. Several large DNA virus-vectored vaccines are currently available, including those that utilize poxviruses. One of the most recognizable of these would be the well-characterized vaccinia virus ^{67–69}.

One of the major problems with viral vectors is the interference of pre-existing immunity against the vector which often precludes the development of immune responses against the heterologous antigens ⁷⁰. In these cases, the vector can only be used once, with a different vector delivering the same antigen being necessary for any type of boost vaccination or delivery of another antigen.

Notably, ORFV-based vectors present several unique properties that may favor their use over current veterinary viral vectors. ORFV has a restricted host range and causes only localized, self-limiting infections ¹⁵. ORFV is also capable of inducing robust immune responses in permissive and non-permissive hosts ⁷¹. Additionally, since ORFV does not induce neutralizing antibody responses, it has the potential to be used multiple times either to boost a previous vaccination or to deliver new antigens against different diseases. The IMPs encoded by ORFV also represent potential sites where foreign DNA could be inserted into the ORFV genome ^{54–56}. These genes are non-essential for ORFV replication and some genes contribute to ORFV virulence in the natural host, which allows the development of an attenuated viral vector for vaccine delivery in animal species.

The use of ORFV as a vaccine delivery platform has been explored. Most of these vaccines use the highly attenuated ORFV strain D1701-V, which was passaged in Vero cell cultures for over 200 passages resulting in multiple gene deletions and rearrangements

throughout the genome. Studies using the ORFV D1701-V describe the insertion of heterologous viral antigens into the VEGF locus ⁷². These studies have shown that ORFV is capable of eliciting immune responses against viruses like influenza A, rabbit hemorrhagic disease virus (RHDV), and rabies virus ^{73–75}. Notably, ORFV D1701 recombinant expressing the pseudorabies virus (PRV) glycoproteins gC and gD or the classical swine fever virus E2 glycoprotein protected swine from PRV and CSFV challenge, respectively ^{76,77}. In this study we evaluated the potential of ORFV vectoring the full length spike (S) protein of porcine epidemic diarrhea virus.

Porcine Epidemic Diarrhea Virus (PEDV)

Porcine epidemic diarrhea virus (PEDV) is an enteric pathogen that causes acute and significant disease in swine ⁷⁸. Infection with PEDV often presents as acute watery diarrhea, vomiting, dehydration, lethargy, and anorexia ⁷⁹. The virus is capable of infecting swine of all ages, but causes the most severe disease in piglets less than 7 days of age resulting in mortality rates as high as 100% in suckling piglets ⁸⁰. Death associated with PEDV is often occurs due to dehydration resulting from severe watery diarrhea, the hallmark symptom for which PEDV was named ^{81–83}. PEDV spreads via the fecal-oral route and is extremely infectious and transmissible, often quickly spreading from one farm to another ^{84,85}. It has also been suggested that PEDV can remain infectious and be transported via the aerosolized route ⁸⁶. Since its initial discovery in the 1970's in Europe, PEDV remained endemic in Asia, causing significant economic losses to the swine industry ⁸⁰. In recent years, the first cases of PEDV were reported in the United States. Within its first year of emergence in the U.S., PEDV was responsible for the death of approximately 7 million piglets (~10% of the domestic pig population) ⁸⁷.

Virus structure and properties

PEDV is a member of the genera *Alphacoronavirus* in the family *Coronaviridae*. It has a genome approximately 28 kb in size consisting of four structural proteins: Spike (S), membrane (M), envelope (E), and nucleocapsid (N) ⁸⁸. PEDV also encodes for 16 additional non-structural proteins, many of which have been suggested to serve important functions in evading host anti-viral responses ⁸⁹. Of the structural proteins, the S protein is a type I glycoprotein of 1,383 amino acids in length with a predicted molecular weight ranging from 180-220 kDa ⁸⁰. The PEDV S has been characterized as playing several critical rolls in virus attachment and entry into cells, in addition to being the primary target for anti-PEDV neutralizing antibodies ⁹⁰. Therefore the PEDV S protein has been targeted for the development of recombinant PEDV vaccines ⁹⁰.

Pathogenesis

Following infection, PEDV replicates in intestinal enterocytes ⁹¹. These cells express abundant amounts of the porcine aminopeptidase N receptor which served as a receptor for PEDV ⁹². Binding is facilitated by the PEDV S protein, which then cleaves into subunits S1 and S2, causing a conformational change in the receptor and internalization of the virus by the cell ⁹³. Replication of PEDV in enterocytes leads to cell lysis, causing villous atrophy and subsequent destruction of the brush border ⁹⁴. Due to the importance of these cells in digestion and nutrient absorption, their destruction by PEDV often leads to malabsorption and severe, acute watery diarrhea ⁹¹. Replicating virus is then shed in the feces where it is spread to other animals ⁹⁵.

Despite its ability to cause disease in swine of all ages, PEDV has notably high mortality rates in suckling piglets. It remains unclear as to why PEDV causes higher

mortality rates in suckling pigs when compared to older pigs ⁸³. Physiological factors associated with the age of the pigs may play important roles, specifically the slower rate of enterocyte regeneration in young pigs ⁹⁶. Although mortality is rare in older animals, there is still considerable morbidity associated with PEDV infection.

Prevention and Vaccines

Vaccination remains the most efficient and cost-effective method of preventing or controlling viral diseases ⁹⁷. Development of vaccines for enteric pathogens like PEDV, however, remains a major challenge, especially because of the need for lactogenic immunity ^{98,99}. One of the earliest and most crude strategies to preventing further PEDV infection was exposure of pregnant sows to live, infectious virus ⁸⁷. Older animals typically are not susceptible to PEDV associated mortality and will recover from the disease ⁸². Sows with previous exposure to PEDV are able to transfer PEDV sIgA antibodies passively to their offspring in the colostrum, effectively protecting them from disease ⁹⁸. Both IgG and IgA are secreted in colostrum, with 60% actually being IgG, yet only the J chain-containing IgA is stable and resists degradation in the gut ¹⁰⁰. Exposure and subsequent immunity to PEDV can be achieved by exposing animals to feces or minced intestines of infected animals ¹⁰¹. This strategy is extremely crude and has several negative implications, especially the idea of using virulent virus on healthy animals, as well as the potential for its spread to other animals and farms and the potential to expose animals to other virulent and damaging diseases ¹⁰². To date, one of the best strategies for preventing PEDV disease comes in the form of taking proper sanitary and hygiene precautions to prevent spread of the highly infectious virus.

Most current PEDV vaccines have been developed for strains circulating in Asia ⁸⁰. Since PEDV emergence in Asia, variants of the first PEDV strain CV777 obtained in 1977 in Europe in either inactivated or attenuated forms. Many of these vaccines were developed by passing the virus multiple times in cell culture, leading to significant mutation accumulation and a genome that can often be significantly different from the original ¹⁰³. Two commonly used viruses are the Korean KPEDV-1 strain, that was attenuated by passaging the virus 93 times in Vero cells ¹⁰⁴, and the Japanese P-5V strain ¹⁰⁵. Both strains are considered to be moderately efficient producers of lactogenic immunity, meaning they are capable of passive immunization of suckling piglets ¹⁰⁵.

Other strategies exist that target the manner in which mucosal immunity is generated, specifically by delivering the vaccine by oral routes. A Korean vaccine using a highly passaged attenuated DR13 strain of PEDV saw dose dependent levels of lactogenic immunity and passive protection when animals were inoculated via the oral route ¹⁰⁶. The vaccine protected 25% of the pigs when given at low doses and up to 50% when given at doses that were 20 times higher. Despite this moderate efficacy, the vaccine failed to inhibit or decrease levels of virus shedding, an important aspect to consider when developing vaccines.

Another oral-vaccination strategy exists incorporating the use of transgenic plants to produce immunogenic PEDV proteins ⁹⁸. In one study, transgenic tobacco plants expressing the PEDV S protein were able to induce neutralizing antibodies in mice when the plant was administered orally. This mechanism could prove extremely promising as it could be both cost effective and mimics the natural mechanism of PEDV exposure, triggering mucosal immunity at sites most likely to encounter live PEDV upon challenge.

In recent years, pork producers have been seeing a decrease in the already questionable efficacy of PEDV vaccines. Some suggest that this is the result of the recent emergence of genetically different, more virulent strains of PEDV ¹⁰⁷, while others contest that the recent spotlight on PEDV has simply exposed their already mediocre efficacy. One inherent problem is based on the methods by which these vaccines are developed, which currently are either by inactivation or attenuation. These two methods are preferential, as they are easy and cost effective to produce ¹⁰⁸. Inactivated vaccines often lack in efficacy due to their lack of replication and stimulation of robust local mucosal immunity ¹⁰⁹. Whereas the attenuation process may lead to a vaccine that is too attenuated, thus eliciting inefficient immune responses. Another concern of live-attenuated strains of PEDV is their potential to possibly revert to a highly virulent phenotype. Studies conducted on recently emerging infectious strains of PEDV have suggested their derivation from an attenuated vaccine; the mutations having caused them to actually become more virulent ¹⁰⁷.

For these reasons, it is paramount that new PEDV vaccines are developed which utilize new and emerging antigen delivery mechanisms, with specific focus on the induction of mucosal immunity and targeting of immunodominant, protective epitopes. There are several ways in which mucosal immunity can be targeted and continued research is being made to improve these methods ¹¹⁰. As far as immunodominant proteins go, the PEDV S protein has been well characterized as the primary target for neutralizing antibodies ¹¹¹. Further research has gone to show several important B-cell epitopes on the PEDV S ¹¹². Therefore, the PEDV S is one of the most promising candidates in terms of developing new recombinant PEDV vaccines. As previously stated, researchers have already

experimentally expressed PEDV-S in transgenic tobacco plants which successfully protected mice from PEDV infection ⁹⁸.

Other vaccination strategies, including the expression of recombinant PEDV S or its delivery by live-virus-vectors, may hold the key for designing functional and efficacious PEDV vaccines. One of the first U.S. licensed vaccines, developed by Harris Vaccine Inc., utilized an alphavirus vector to vaccinate against PEDV ¹¹³. There has also been the development of inactivated vaccines using strains isolated from infected pigs in the United States ^{114,115}. Regardless of the mechanism of delivery, the severity of disease caused by PEDV make the development of a successful vaccine critical for preventing disease in swine and significant economic damage to the pork-producing industry.

Chapter 2: Immunogenicity of a recombinant parapoxvirus expressing the spike protein
of porcine epidemic diarrhea virus

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Abstract

The parapoxvirus Orf virus (ORFV) has long been recognized for its immunomodulatory properties in permissive and non-permissive animal species. Here, a new recombinant ORFV expressing the full-length spike (S) protein of porcine epidemic diarrhea virus (PEDV) was generated and its immunogenicity and protective efficacy were evaluated in pigs. The PEDV S was inserted into the *ORFV121* gene locus, a novel immunomodulatory gene that inhibits activation of the nuclear factor- κ B (NF- κ B) signaling pathway and contributes to ORFV virulence in the natural host. The recombinant ORFV-PEDV-S virus efficiently and stably expressed the PEDV S protein in cell culture *in vitro*. Intramuscular (IM) prime-boost immunizations with the recombinant ORFV-PEDV-S in 3-week-old pigs elicited robust serum IgG, IgA and neutralizing antibody responses against PEDV. Additionally, IM immunization with the recombinant ORFV-PEDV-S virus protected pigs from clinical signs of porcine epidemic diarrhea (PED) and reduced virus shedding in feces upon challenge infection. These results demonstrate the suitability of *ORFV121* gene locus as an insertion site for heterologous gene expression and delivery by ORFV-based viral vectors. Additionally, the results provide evidence of the potential of ORFV as a vaccine delivery vector for enteric viral diseases of swine. This study may have important implications for future development of ORFV-based viral vectored vaccines for swine.

Introduction

Orf virus (ORFV) is the type member of the genus *Parapoxvirus* of the family *Poxviridae*⁶. ORFV is a highly epitheliotropic virus, and keratinocytes and epithelial cells in the oral mucosa are the most important - if not the only - cell type to support ORFV replication in natural hosts⁵. The ORFV genome consists of a double-stranded DNA molecule of approximately 138 kbp in length and contains 131 putative genes². ORFV has been long known for its immunomodulatory properties^{71,116}. Many genes with immunomodulatory functions have been identified in the ORFV genome, including a homologue of interleukin 10 (IL-10)⁴⁷, a chemokine binding protein (CBP)¹¹⁷, a secreted inhibitor of granulocyte-monocyte colony-stimulating factor (GM-CSF) and IL-2⁴³, a vascular endothelial growth factor (VEGF)³⁸, an interferon (IFN)-resistance gene¹¹⁸, and, more recently, three inhibitors of the nuclear factor- κ B (NF- κ B) signaling pathway (*ORFV002*, *ORFV024* and *ORFV121*)^{54–56}. Among these, the IL-10 homologue, the VEGF gene and the NF- κ B inhibitor *ORFV121* have been shown to contribute to ORFV virulence in the natural host^{49,55,119}.

ORFV has been historically used as a preventive or therapeutic agent in veterinary medicine¹¹⁶. The potential of ORFV as a recombinant vaccine delivery vector has been explored, and recombinant ORFV vectors based on the highly attenuated ORFV strain D1701 have been shown to induce protective immunity against several viral diseases in permissive and non-permissive animal species^{72–77,120–122}. Notably, ORFV D1701-based recombinants expressing the Pseudorabies virus (PRV) glycoproteins gC or gD induced protective immunity against PRV infection in pigs⁷⁶, while a recombinant ORFV expressing the classical swine fever virus (CSFV) E2 glycoprotein protected swine against

intranasal challenge with a virulent CSFV strain ⁷⁷. These studies demonstrate the efficacy of ORFV-based vectors in eliciting protective immune responses in swine. In the present study, the potential of ORFV as a vaccine delivery vector for enteric viral diseases of swine was investigated. The porcine epidemic diarrhea virus (PEDV) spike (S) glycoprotein was used as a model antigen to evaluate the immunogenicity and protective efficacy ORFV-based vectors in pigs.

Porcine epidemic diarrhea virus (PEDV), a member of the genus *Acoronavirus* of the family *Coronaviridae*, causes severe enteric disease (porcine epidemic diarrhea; PED) in pigs ^{123,124}. PEDV infects pigs of all ages, producing high mortality rates (50-100%) in suckling piglets and weight loss due to diarrhea in older animals ¹²³. The virus replicates primarily in enterocytes of the small intestines leading to villous atrophy and malabsorptive diarrhea followed by electrolyte imbalance, metabolic acidosis and death ⁸³. Characteristic clinical signs of PED include watery diarrhea, vomiting, anorexia, dehydration and death ¹²⁵. In older pigs, the disease is usually milder resulting in low mortality rates ¹²⁵.

The PEDV genome is a large (~28 Kb) positive sense RNA molecule that contains six open reading frames (ORFs), encoding the replicase proteins (pp1a and pp1ab), four structural proteins (Spike [S], envelope [E], membrane [M], and nucleoprotein [N]) and one accessory protein (ORF3) ^{80,125}. Among the structural proteins, the S protein is the major envelope glycoprotein responsible for virus attachment and entry ^{112,126,127}. Given its critical function in attachment and entry, the S glycoprotein is the main target for neutralizing antibodies (NA) against PEDV ¹²⁷⁻¹³⁰, with several neutralizing epitopes being mapped to this glycoprotein ^{112,126-128,131}.

Here a novel recombinant ORFV expressing the full-length PEDV S protein (ORFV-PEDV-S) was generated, and its immunogenicity and protective efficacy were evaluated in pigs. The PEDV S coding sequences were inserted into the *ORFV121* gene locus, a recently characterized immunomodulatory gene of ORFV that contributes to the virus virulence in the natural host ⁵⁵. Results from immunization studies in pigs show that intramuscular (IM) immunization with the ORFV-PEDV-S elicited S-specific IgG, IgA and neutralizing antibody responses. Notably, animals immunized with the ORFV-PEDV-S via the IM route were protected from clinical signs of PED, and presented reduced virus shedding in feces after oral challenge with a virulent PEDV strain.

Materials and methods

Cells and viruses. Primary ovine fetal turbinate- (OFTu), porcine kidney- (PK-15; ATCC PTA-8244) and Vero-76 cells (ATCC® CRL-1587™) were cultured at 37°C with 5% CO₂ in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and containing penicillin (100 U/mL), streptomycin (100 µg/mL) and gentamicin (50 µg/mL).

ORFV strain IA82 ² was used as the parental virus to construct the recombinant Orf virus expressing the PEDV S protein (ORFV-PEDV-S) and in all experiments involving the use of wild type ORFV. Wild type and recombinant ORFV viruses were amplified in primary OFTu cells. PEDV strain USA/CO/2013 (CO13) was obtained from the National Veterinary Services Laboratory (NVSL) and propagated in Vero-76 cells in the presence of 1.5 µg/mL TPCK treated trypsin (Sigma Aldrich, St. Louis, MO).

Construction of ORFV-PEDV-S recombination plasmid. The full-length coding sequence of the spike gene of PEDV strain CO13 (GenBank accession no. KF267450) was analyzed, and restriction endonuclease sites required for insertion into the ORFV genome (*ORFV121* locus⁵⁵) were removed through silent nucleotide substitutions. In addition, early poxviral transcription termination signals (TTTTTNT) present within the coding sequence of PEDV S were removed by introducing silent nucleotide mutations. Coding sequences of the His-tag epitope (6xHis) were added to the 5' and 3' ends of the S coding sequence. The sequence of the VV.7.5 early/late poxviral promoter¹³² was added to the 5' end of the PEDV S coding sequence. Additionally, HindIII and SalI restriction sites were added to the 5' and 3' ends of the VV7.5-PEDV-S construct, respectively. A single DNA fragment containing the full length PEDV S coding sequences under the control of the VV.7.5 early/late poxviral promoter was chemically synthesized (GenScript®, Piscataway, NJ) and subcloned into the poxviral transfer vector pZippy-EGFP¹³³ using HindIII and SalI restriction enzymes (pZGFP-PEDV-S).

To insert the PEDV-S coding sequences into the *ORFV121* genome locus⁵⁵, a recombination cassette was constructed. *ORFV121* left (LF, 1016 bp) and right (RF, 853 bp) flanking regions were PCR amplified from the ORFV strain IA82 genome with primers 121LF-Fw(SpeI)-5'-ATTCTTATGCGGCCGCGCAGCACTGCTCGGAGGAGTGCTC-3'; 121LF-Rv (HindIII)-5'-CAGAATTCGCAAGCTTGTTGTGTGGGCCACAGAGTTGAG-3'; 121RF-Fw (NotI)-5'-ATTCTTATGCGGCCGCGGAGCACTGCTCGGAGGAGTGCT-3'; and 121RF-Rv (BglII)-5'-CAGAATTCGCAGATCTATCATGCGCAGCGACGACATCATC-3' and cloned into the vector pZGFP-PEDV-S resulting in the

recombination vector pZGFP-121PEDV-S. Correct cloning of ORFV121 LF and RF and of PEDV-S were confirmed by restriction enzyme analysis.

Generation and characterization of the ORFV-PEDV-S recombinant virus. The full length PEDV Spike coding sequences were inserted into the *ORFV121* locus⁵⁵ of the ORFV genome by homologous recombination between the parental ORFV strain IA82 and the recombination cassette pZGFP-121PEDV-S. OFTu cells cultured in 6-well plates were infected with OV-IA82 (multiplicity of infection [MOI] = 1) and 3 h later transfected with 2 µg of pZGFP-121PEDV-S DNA using Lipofectamine 3000 (Life Technologies) according to the manufacturer's instructions. At 72 h post-infection/transfection cell cultures were harvested, subjected to three freeze-and-thaw cycles and cell lysates used for recombinant virus selection by limiting dilution followed by plaque assay. Briefly, OFTu cells cultured in 96-well plates were infected with 10-fold serial dilutions of the cell lysates (10^{-1} to 10^{-3}), incubated at 37°C for 72 h, and screened under a fluorescence microscope. Wells containing viral foci expressing the green fluorescent protein (GFP) were harvested and subjected to one additional round of limiting dilution. GFP positive wells from the second limiting dilution were subjected to plaque purification. OFTu cells cultured in 6-well plates were infected with 10-fold serial dilutions (10^{-1} to 10^{-3}) of cell lysates from GFP positive wells (obtained during the limiting dilutions selection) and overlaid with culture medium containing 0.5% agarose (SeaKem GTC agarose, Lonza Inc., Aretta, GA). Fluorescent plaques were subjected to five additional rounds of plaque purification. The presence of PEDV-S and absence of *ORFV121* sequences in the purified recombinant virus were confirmed by PCR screening. Primers used for PCR amplification of PEDV-S sequences were PEDV-intS-Fw-5'-CGTGGTGGGTTTGGTTGATT-3' and PEDV-intS-

Rv-5'-CTGCACGTGGACCTTTTCAA-3'; and 121int-Fw-5'-GGCGGACTAC CAGAGACATC-3' and 121int-Rv-5'-GTCTTCCGGGATGTCGTAGA-3', respectively. PCR amplicons were analyzed by electrophoresis in 1% agarose gels. Insertion and integrity of the PEDV full-length spike sequences were confirmed by whole genome sequencing using the Nextera XT DNA library preparation kit (Illumina, San Diego, CA) followed by sequencing on the Illumina Mi-Seq sequencing platform (Illumina, San Diego, CA).

Immunofluorescence. Expression of PEDV S by the ORFV-PEDV-S recombinant virus was assessed by immunofluorescence assay. OFTu cells were infected with the ORFV-PEDV-S recombinant virus (MOI = 1) and fixed with 3.7% formaldehyde at 24 h post-infection. After fixation cells were washed three times with phosphate buffer saline (PBS) and permeabilized with 0.2% PBS-Triton X100 for 10 min at room temperature (RT). Unpermeabilized cells were kept as controls to assess expression of PEDV S on the membrane of ORFV-PEDV-S infected cells. Cells were washed three times with PBS and incubated with a PEDV S specific mouse monoclonal antibody (SD37-11) for 1 h at room temperature (RT). After primary antibody incubation, cells were washed as above and incubated with goat anti-mouse IgG (H+L) secondary antibody (Alexa Fluor® 594 conjugate; Life Technologies, Carlsbad, CA) for 1 h at RT. Cells were washed three times with PBS and visualized under a fluorescence microscope.

Western blot. Expression of PEDV S by the ORFV-PEDV-S recombinant virus was assessed by Western blot. OFTu cells cultured in 6-well plates were infected ORFV-PEDV-S recombinant virus (MOI = 10) and harvested at 48 h post-infection. Cells infected with parental ORFV strain IA82 were used as controls. Cells were lysed with M-PER

mammalian extraction reagent (Thermo Scientific, Waltham, MA) containing protease inhibitors (RPI, Mount Prospect, IL). Fifty micrograms of whole cell protein extracts were resolved by SDS-PAGE in 7% acrylamide gels and transferred to nitrocellulose membranes. Blots were incubated with 5% non-fat dry milk TBS-Tween 20 (0.1%; TBS-T) solution for 1 h at RT and probed with 6x-His epitope tag antibody (His.H8, Thermo Scientific, Waltham, MA) overnight at 4°C. Blots were washed three times with TBS-T for 10 min at RT and incubated with a goat anti-mouse IgG-HRP conjugate secondary antibody for 2 h at RT. Blots were washed three times with TBS-T for 10 min and developed by using a chemiluminescent substrate (Clarity, ECL; Bio-Rad, Hercules, CA).

Growth curves. Replication properties of ORFV-PEDV-S recombinant virus were assessed *in vitro*. OFTu and PK15 cells were cultured in 6-well plates, inoculated with ORFV-PEDV-S (MOI = 0.1 [multi-step growth curve] and MOI = 10 [single-step growth curve]) and harvested at various time points post-infection (6, 12, 24, 48, and 72 h p.i.). Virus titers were determined on each time point using the Spearman and Karber's method and expressed as tissue culture infectious dose 50 (TCID₅₀)/mL.

Antigens for ELISAs. A truncated form of PEDV S1 protein was expressed as a recombinant protein in *E. coli*, and used in indirect ELISAs to assess antibody responses in animals immunized with the ORFV-PEDV-S virus. A fragment of the PEDV S1 protein corresponding to nucleotides 1891 to 2400 was amplified from the genome of PEDV strain CO13 using standard reverse transcriptase and PCR amplification methods. Primers used for PCR amplification were PED-SPikS1-1891(BamHI)-F-5'-CGCGGATCCACGCCTAAACCATTTGAAG-3' and PED-SpikS1-2400(XhoI)R-5'-CACACTCGAGGTAAAGCTGTAAATATTCTGTCC-3'. The PCR amplicon was cloned into the pET-28a

eukaryotic expression plasmid (EMD Millipore – Novagen, Billerica, MA) using the restriction enzymes indicated on each primer sequence (underlined). The recombinant S1 protein was expressed in *E. coli* as a 6X histidine-tagged fusion protein¹³⁴ and purified using nickel-charged agarose resin (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Affinity purified recombinant protein was refolded and used as antigen on indirect ELISAs. DNA sequencing was used to confirm the identity and in frame cloning of PEDV S1 with 6X His-tag.

Animal immunization-challenge studies. The immunogenicity and protective efficacy of the ORFV-PEDV-S recombinant virus were assessed in pigs. The efficacy of the vector was evaluated by two immunization routes: transcutaneous (TC) and intramuscular (IM). The TC route was explored here because it has been shown to induce mucosal and systemic immunity in mice and humans (40), and because of the natural tropism of ORFV for skin keratinocytes⁵. Sixteen 3-week-old pigs, seronegative for PEDV, were randomly allocated to four experimental groups as follows: Group 1, sham-immunized/mock-challenged ($n = 4$); Group 2, ORFV-PEDV-S-immunized/PEDV challenged ($n = 4$); Group 3, ORFV-PEDV-S-immunized/PEDV challenged ($n = 4$); and Group 4, sham-immunized/PEDV challenged ($n = 4$) (Table 1). Animals from groups 1 and 4 were immunized with a control ORFV vector expressing GFP (ORFV-GFP) and received the vaccine via TC (1 mL) and IM routes (1 mL). Animals from group 2 were immunized with the ORFV-PEDV-S recombinant virus via the TC route, while animals in group 3 were immunized by the IM route. Intramuscular immunization was performed by injection of 2 mL of a virus suspension containing $10^{7.38}$ TCID₅₀/mL in MEM into the neck. For the TC immunization the skin of the inguinal region was scarified with a sterile scalpel blade and the virus

suspension containing $10^{7.38}$ TCID₅₀/mL in MEM was applied topically to the scarified skin area using a sterile cotton swab (~4 cm²). All animals were immunized on day 0 and received two booster immunizations on days 21 and 45 post-primary immunization. Serum samples were collected on days 0, 7, 14, 21, 35, 42, 49, 53, 56 and 60 post-immunization.

The protective efficacy of recombinant ORFV-PEDV-S virus was assessed following challenge infection with PEDV. On day 60 post-immunization, animals from Group 1 received 2 mL of MEM orally (mock-challenge), while animals from groups 2, 3 and 4 were challenged orally with a virus suspension containing 2×10^5 TCID₅₀ of PEDV strain CO13. Animals were monitored daily for clinical signs of PED. Clinical signs were recorded and individual daily scores assigned to all animals based on the following criteria: 0 = normal feces, 1 = pasty feces, 2 = moderate diarrhea (semi-liquid), 3 = diarrhea (liquid), 4 = severe diarrhea (very liquid), 5 = watery diarrhea (profuse diarrhea) ¹³⁷. Mean daily group scores were calculated and compared among different treatment groups. Fecal swabs were collected on day 0, 3, 5, 7, 9, 11 and 14 post-challenge to assess virus shedding in feces. Serum samples were collected on days 0, 3, 7, 10 and 14 post-challenge to evaluate humoral and cell mediated responses. All animals were euthanized on day 14 post-challenge. Animal immunization-challenge studies were conducted at SDSU Animal Resource Wing (ARW), following the guidelines and protocols approved by the SDSU Institutional Animal Care and Use Committee (IACUC approval no. 15-063A).

Antibody isotype ELISAs. PEDV specific IgG and IgA antibody responses elicited by immunization with the recombinant ORFV-PEDV-S virus were assessed by S1 indirect ELISAs, while responses post-challenge infection were assessed by the S1 and nucleoprotein (N) ¹³⁴ ELISAs. Optimal assay conditions (amount of antigen, serum and

secondary antibody dilutions) were determined by a checkerboard titration. Polystyrene microtiter plates (Immunolon 1B, Thermo Scientific, Waltham, MA) were coated with the appropriate antigen (S1, 100 ng/well; and N, 25 ng/well¹³⁴) in bicarbonate/carbonate coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) in alternate wells. After incubation at 37°C for 1 h, plates were washed four times with PBS tween 20 (PBS-T, 0.05%) and blocked overnight at 4°C with PBS-T 5% non-fat dry milk. Blocking reagent was removed and plates washed three times with PBS-T (300 µl). Test and control serum samples were diluted (1:50) in PBS-T 5% non-fat dry milk, and 100 µl of diluted samples were added to paired coated and uncoated control wells and incubated at room temperature for 1 h. Unbound antibodies were washed with PBS-T (three times) and plates incubated with biotinylated secondary antibodies against swine IgG or IgA (Bethyl Laboratories, TX) followed by incubation with streptavidin-HRP conjugate (Pierce, Rockford, IL). Reactions were developed with 3,3',5'5' – tetramethylbenzidine substrate (TMB) (KPL Inc., Gaithersburg, MA) and OD values determined at 450 nm using a microplate reader (BioTek Instruments Inc., Winooski, VT). OD values for each test and control samples were normalized to the OD value of uncoated wells and results expressed as sample to positive (S/P) ratios that were calculated as follows: $S/P = \frac{\text{optical density (OD) of sample} - \text{OD of buffer}}{\text{OD of positive control} - \text{OD of buffer}}$. All assay formats were pre-validated at the SD ADRDL using serum samples from animals of known serological status.

Fluorescent focus neutralization assay. Neutralizing antibody responses elicited by immunization with the recombinant ORFV-PEDV-S were assessed by fluorescent focus neutralization assay (FFN) as previously described¹³⁴. Endpoint neutralizing antibody

titers were determined as the highest dilution of serum capable of reducing 90% of PEDV fluorescent foci relative to negative control samples. A FFN titer <20 was considered negative.

Viral RNA extraction and real-time RT-PCR. Viral nucleic acid was extracted from fecal swabs using the MagMAX viral RNA/DNA isolation kit (Life Technologies, Carlsbad, CA) following the manufacturer's instructions. Shedding of PEDV in feces was assessed using a commercial multiplex real-time RT-PCR (rRT-PCR) kit targeting the spike gene of PEDV, and other enteric coronaviruses including transmissible gastroenteritis virus (TGEV) and porcine deltacoronavirus (PDCoV) (EZ-PED/TGE/PDCoV MPX 1.0, Tetracore Inc., Rockville, MD). Genome copy number per mL were determined using the relative standard curve method. All calculations were performed using a 4-parameter logistic regression curve. rRT-PCR tests were performed at the SDSU Animal Disease and Research Diagnostic Laboratory (ADRDL).

Statistical analysis. Statistical analysis was performed using SPSS 14 software. One-way analysis of variance with Tukey HSD multiple comparison test was performed on all groups, using harmonic mean sample size of 4 to account for equal group sizes. Nonparametric Kruskal-Wallis test was used to assess differences in virus shedding between treatment groups. Differences between groups were considered significant at $P < 0.05$.

Results

Generation and characterization of the ORFV-PEDV-S recombinant virus. The full-length spike protein of PEDV was inserted into the *ORFV121*⁵⁵ genome locus of ORFV

by homologous recombination. *ORFV121*⁵⁵, was deleted from the ORFV genome and replaced by a DNA fragment encoding full-length PEDV S protein and the reporter gene encoding the green fluorescent protein (GFP) under the control of individual early/late VV7.5 poxviral promoters (Fig. 1A). The recombinant virus was selected and purified by limiting dilution followed by plaque assays based on expression of the green fluorescence protein (GFP) (Fig. 1C). Deleted *ORFV121* gene sequences were not detected in the purified recombinant virus (Fig. 1B). In contrast, PEDV S sequences were detected in the recombinant virus but not in the wild type ORFV genome (Fig. 1B). Complete genome sequence of the ORFV-PEDV-S recombinant virus confirmed the insertion of the full-length coding sequences of PEDV S (data not shown).

Replication properties of the ORFV-PEDV-S recombinant virus were assessed *in vitro*. No differences in replication kinetics and viral yields were observed when multiple-step or one-step growth curves of ORFV-PEDV-S were compared to those of the wild-type OV-IA82 virus in primary ovine fetal turbinate (OFTu) cell cultures (data not shown). Replication kinetics of ORFV-PEDV-S was also assessed in porcine kidney cells (PK-15). Notably, replication of ORFV-PEDV-S in PK-15 cells was markedly impaired when compared to its replication in natural host OFTu cells (Fig. 3A and 3B). Similar replication kinetics was observed in swine testicle cells (ST, data not shown). These results indicate a marked growth defect for ORFV-PEDV-S in swine cells, demonstrating only minimal virus replication in cells of swine origin.

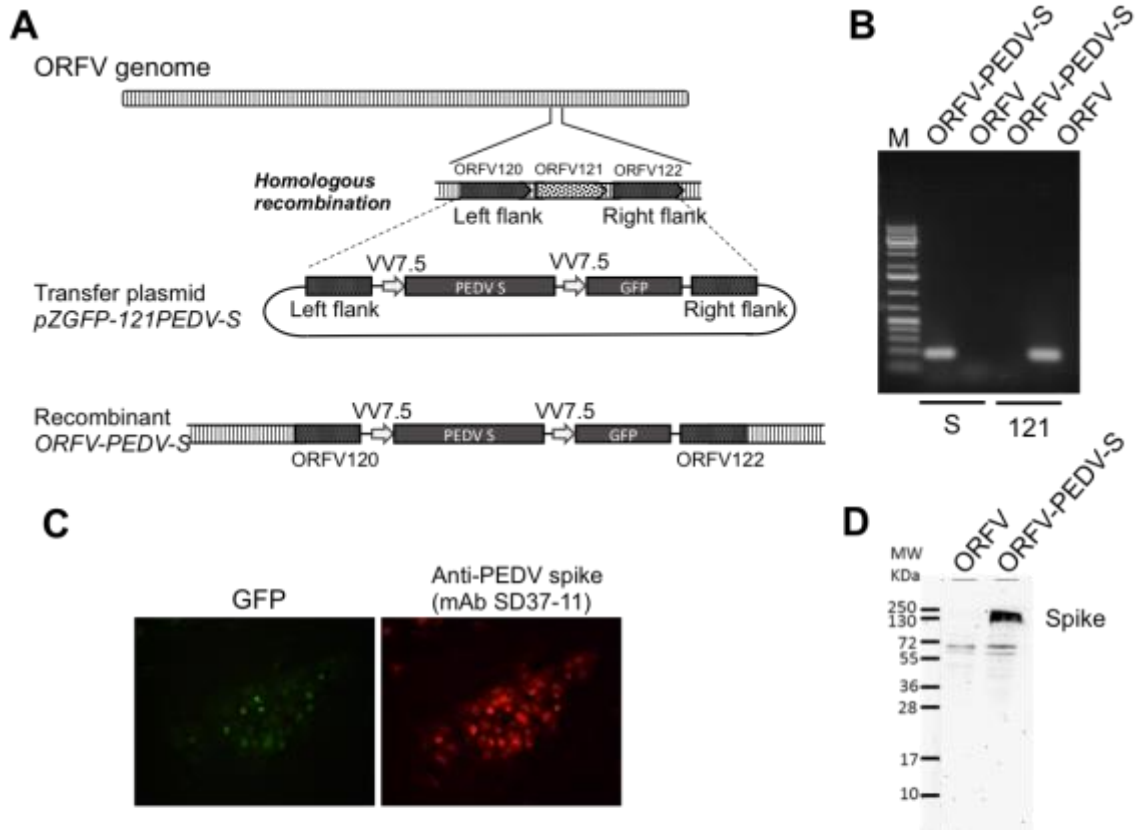


Figure 1. Generation of recombinant ORFV-PEDV-S virus. (A) Schematic representation of the ORFV genome depicting *ORFV121* insertion site and flanking regions (*ORFV120* and *ORFV122*) used to generate the recombinant ORFV-PEDV-S virus. pZGFP-121PEDV-S transfer plasmid containing the full-length PEDV S gene plus the green fluorescent protein as a selection reporter marker under the control of individual early/late VV7.5 poxviral promoters. Recombinant ORFV-PEDV-S genome depicting insertion of the PEDV S and the GFP reporter gene into the *ORFV121* gene locus. (B) Agarose gel (1%) demonstrating PCR amplification of an internal region of the S gene (~150 bp) from the genome of the recombinant ORFV-PEDV-S gene and absence of *ORFV121* gene sequences on the recombinant ORFV-PEDV-S virus. Wild type ORFV DNA was used as a negative and positive control on the PCR amplifications with S-specific and ORFV121 specific primers, respectively. (C) Immunofluorescence assay demonstrating expression of PEDV S in cell cultures infected with the recombinant ORFV-PEDV-S virus. Left panel shows expression of the reporter GFP protein by the recombinant virus. Right panel shows expression of PEDV S detected with a mouse monoclonal antibody against PEDV S. (D) Western blot demonstrating expression of the full-length PEDV S (~150 kDa) by the recombinant ORFV-PEDV-S virus in cell culture *in vitro*. Cell lysates from cells infected with wild type ORFV were used as negative controls. Blot was developed with an anti-Histidine monoclonal antibody.

The recombinant ORFV-PEDV-S expresses PEDV S *in vitro*. Expression of PEDV S by ORFV-PEDV-S recombinant virus was assessed by immunofluorescence and Western blot assays. Expression of PEDV S by the recombinant ORFV-PEDV-S was assessed during virus infection in OFTu cells by using an S-specific monoclonal antibody in an indirect IFA assay. High levels of PEDV S were detected in ORFV-PEDV-S infected cells (Fig.

1C). Similarly, high levels of the full length PEDV S (~150 kDa) were detected in ORFV-PEDV-S infected cells by Western blot assays (Fig. 1D).

Intracellular or surface expression of PEDV S by the recombinant ORFV-PEDV-S was assessed by IFA assays. As shown in Fig. 2A, PEDV S expression was detected in permeabilized (Triton X-100) and in unpermeabilized cells, indicating abundant localization of PEDV S on the surface and intracellular compartment of ORFV-PEDV-S infected cells.

The stability of PEDV S gene inserted into the *ORFV121* locus of the ORFV-PEDV- The stability of PEDV S gene inserted into the *ORFV121* locus of the ORFV-PEDV-S genome was assessed by IFA assays and confirmed by PCR and DNA sequencing following serial passages of the recombinant virus *in vitro*. Expression of PEDV S was consistently detected on ORFV-PEDV-S infected cells after 1, 5 or 10 passages of the recombinant virus in cell culture *in vitro* (Fig. 3b). Additionally, the full length PEDV S was amplified from the recombinant virus on passages 1, 5 and 10 and sequencing of the p.10 full length S revealed 100% identity with the S sequences inserted in the recombinant virus (data not shown). Together, these results demonstrate the stability of PEDV S inserted into the *ORFV121* locus.

Intramuscular (IM) immunization with ORFV-PEDV-S induces serum IgG and IgA antibody responses against PEDV in pigs. The immunogenicity of ORFV-PEDV-S recombinant virus was evaluated in pigs following transcutaneous (TC) or intramuscular (IM) immunizations. Animals were immunized with ORFV-PEDV-S via the TC or IM routes on day 0, and received booster immunizations on days 21 and 45 post-primary

immunization (Table 1). Serological responses elicited against PEDV were monitored by indirect S1 IgG- and IgA-isotype ELISAs. All animals immunized via the IM route with the recombinant ORFV-PEDV-S virus developed IgG and IgA antibody responses against PEDV S (Group 3; Fig. 4A and 4B), whereas no antibody responses were detected in the animals immunized via the TC route (Group 2) or in animals from control groups (Group 1 and Group 4), immunized with ORFV-GFP vectors (Group 1 and 4; Fig. 4A and 4B). Antibodies were first detected on animals from Group 3 (IM) on day 28 post-immunization (p.i.), following the booster immunization on day 21 (Fig. 4A and 4B). Notably, while a second booster immunization elicited anamnestic antibody responses in animals immunized by the IM route, no serological responses were detected in animals immunized by the TC route (Fig. 4A and 4B). Similar results were observed when serological responses were monitored using a whole virus (PEDV) indirect ELISA (data not shown). These results demonstrate that IM immunization with ORFV-PEDV-S elicits robust antibody (IgG and IgA) responses in immunized pigs.

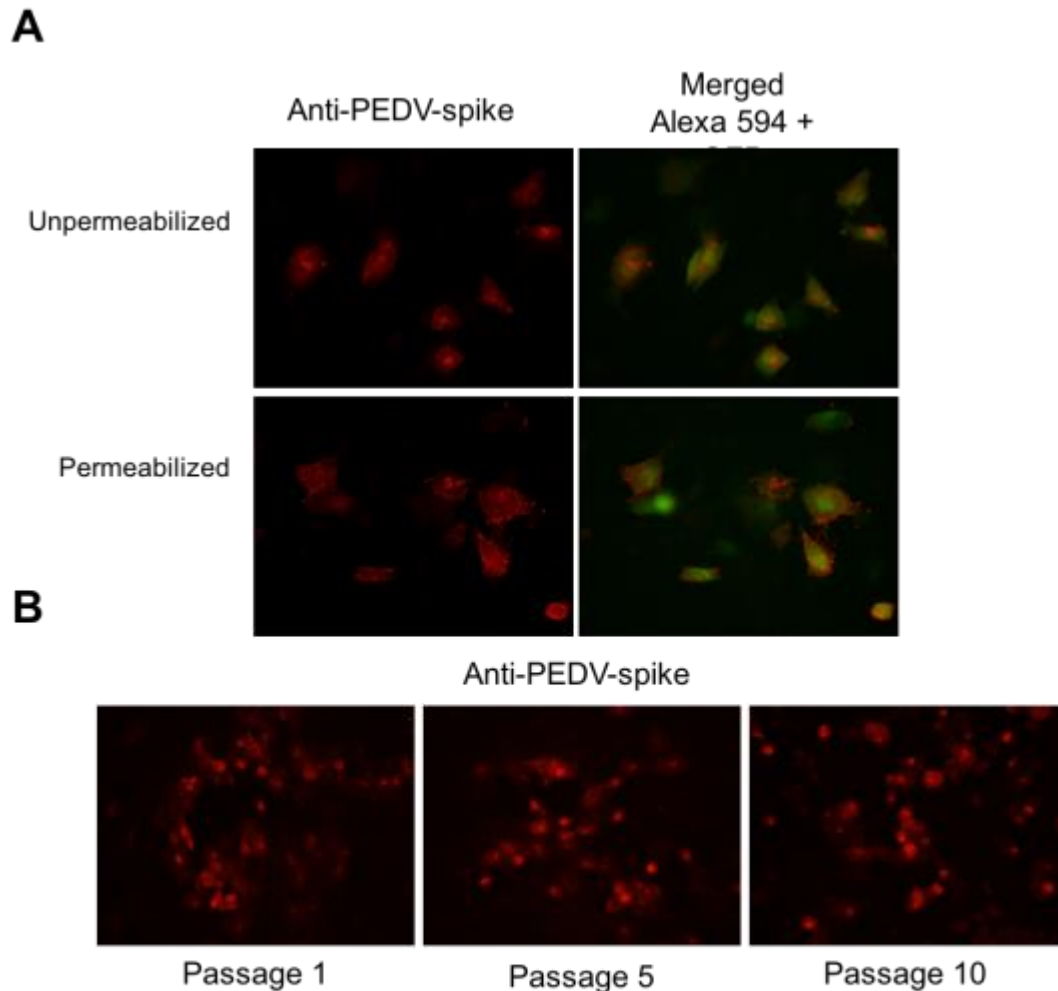


Figure 2. Expression of PEDV S by the recombinant ORFV-PEDV-S. (A) Immunofluorescence assay performed in primary OFTu cells infected (MOI = 1) with the recombinant ORFV-PEDV-S. Cells were fixed with formaldehyde (3.7%) at 24 h post-infection, permeabilized or not with Triton X-100, stained with anti-PEDV-S monoclonal antibody (SD37-11) and visualized under a fluorescence microscope. Left panels show expression of the GFP reporter protein. (B) Immunofluorescence assay showing expression of PEDV S by the recombinant ORFV-PEDV-S after serial passages in cell culture. Primary OFTu cells were infected with passages 1, 5 or 10 of ORFV-PEDV-S (MOI = 3) and fixed, stained and permeabilized as described in A.

IM immunization with ORFV-PEDV-S induces neutralizing antibody responses against PEDV in pigs. The ability of ORFV-PEDV-S to induce neutralizing antibody (NA) responses against PEDV was assessed using an FFN assay ¹³⁴. Similar to the serological responses detected with the IgG and IgA isotype ELISAs, virus neutralization assays revealed that IM immunization with ORFV-PEDV-S elicited NA responses against PEDV

in all immunized animals (Fig. 4C). No NA responses were detected in animals immunized via the TC route or in control sham-immunized animals (Group 1 and 4; Fig. 4C).

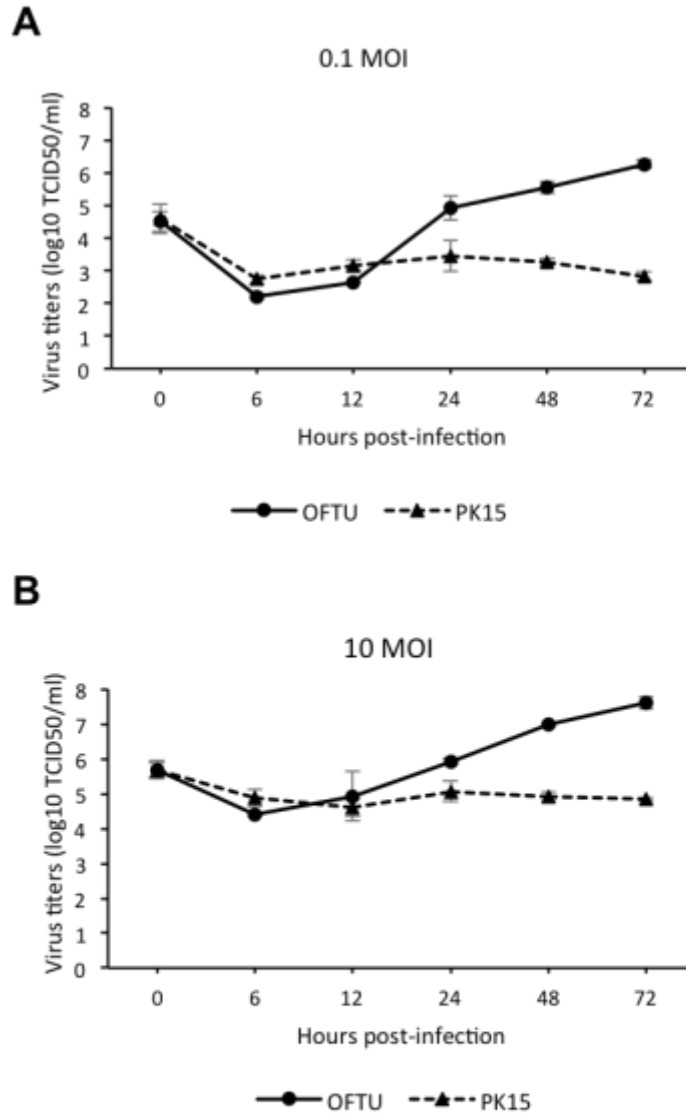


Figure 3. Replication properties of the recombinant ORFV-PEDV-S. (A) Multi-step growth curve of the recombinant ORFV-PEDV-S in primary OFTu and porcine kidney (PK15) cells. (B) Single-step growth curve of the recombinant ORFV-PEDV-S in primary OFTu and porcine kidney (PK15) cells. Cells were collected at indicated time points and virus titers determined by the Spearman and Karber's method and expressed as log₁₀ tissue culture infections dose 50 (TCID₅₀) per mL. Error bars represent standard error of the mean (SEM) calculated based on the results of three independent experiments.

Table 1. Experimental design of animal immunization-challenge infection.

Group ^a	Vector construct	Route	Immunization days	Challenge infection ^d	
				Inoculum	Day p.i.
1	ORFV-GFP	TC ^b + IM ^c	0, 21, 45	MEM	60
2	ORFV-PEDV-S	TC	0, 21, 45	PEDV CO13	60
3	ORFV-PEDV-S	IM	0, 21, 45	PEDV CO13	60
4	ORFV-GFP	TC + IM	0, 21, 45	PEDV CO13	60

^aEach group consisted of four three-week-old weaned piglets.

^bTC: transcutaneous. Virus suspension was instilled on a 4 cm² area of scarified skin.

^cIM: intramuscular. Virus suspension was injected intramuscularly.

^dEach animal was challenged orally with a virus suspension containing 2×10^5 TCID₅₀ of PEDV strain CO13.

IM immunization with ORFV-PEDV-S protects pigs from clinical PED. To evaluate the protective efficacy of the recombinant ORFV-PEDV-S, pigs from Groups 2, 3 and 4 (Table 1) were challenged orally with the virulent PEDV strain CO13 on day 60 p.i. (2×10^5 TCID₅₀/animal). Clinical signs of PED and virus shedding in feces were monitored after challenge infection. Notably, characteristic clinical signs of PEDV infection were observed in 2/4 (50%) animals from the sham-immunized group (Group 4), and in 3/4 (75%) animals from the TC immunized group (Group 2), while no animals immunized via the IM route (0/4) (Group 3) developed clinical signs of PED. Daily average clinical scores were recorded for each group, and are presented in Fig. 5A. Control sham-immunized animals (Group 4) and TC immunized animals (Group 2) presented significantly higher clinical scores when compared to sham-immunized non-challenged animals (Group 1) or to ORFV-PEDV-S immunized (IM) PEDV challenged animals (Group 3) (Fig. 5).

Virus shedding in feces was evaluated after challenge infection using rRT-PCR. Rectal swabs collected from all animals on days 0, 3, 5, 7, 9, 11 and 14 post-challenge (p.c.) infection were tested by PEDV rRT-PCR and the duration of virus shedding as well as PEDV genome copy numbers were compared between treatment groups. All challenged animals (Groups 2, 3 and 4) shed PEDV in feces, while no virus shedding was detected in non-challenged animals (Group 1) throughout the experiment (Table 2). Notably, both duration and magnitude of PEDV shedding were markedly decreased in animals from Group 3, immunized via the IM route with the ORFV-PEDV-S virus (Table 2; Fig. 5B). While $\frac{3}{4}$ animals in Group 2 (75%) and 4/4 animals in Group 4 (4/4; 100%) were positive on day 3 p.c., only $\frac{1}{4}$ animal in Group 3 (25%) was positive for PEDV (Table 2). Animals in Group 3 shed PEDV in feces between days 7 and 9 p.c.; however, they ceased shedding virus earlier than animals from Groups 2 and 4 (Table 2). While on day 11 p.c. only $\frac{1}{4}$ (25%) animal from Group 3 was positive, all 4/4 (100%) animals in Groups 2 and 4 were still shedding PEDV. Quantitation of PEDV genome copy numbers in fecal swabs revealed a lower amount of the virus being shed in feces by animals from Group 3, compared to animals from Groups 2 and 4 (Fig. 5B). Together these results indicate that immune responses elicited by IM immunization with the recombinant ORFV-PEDV-S led to protection from clinical PED and reduced virus shedding in feces.

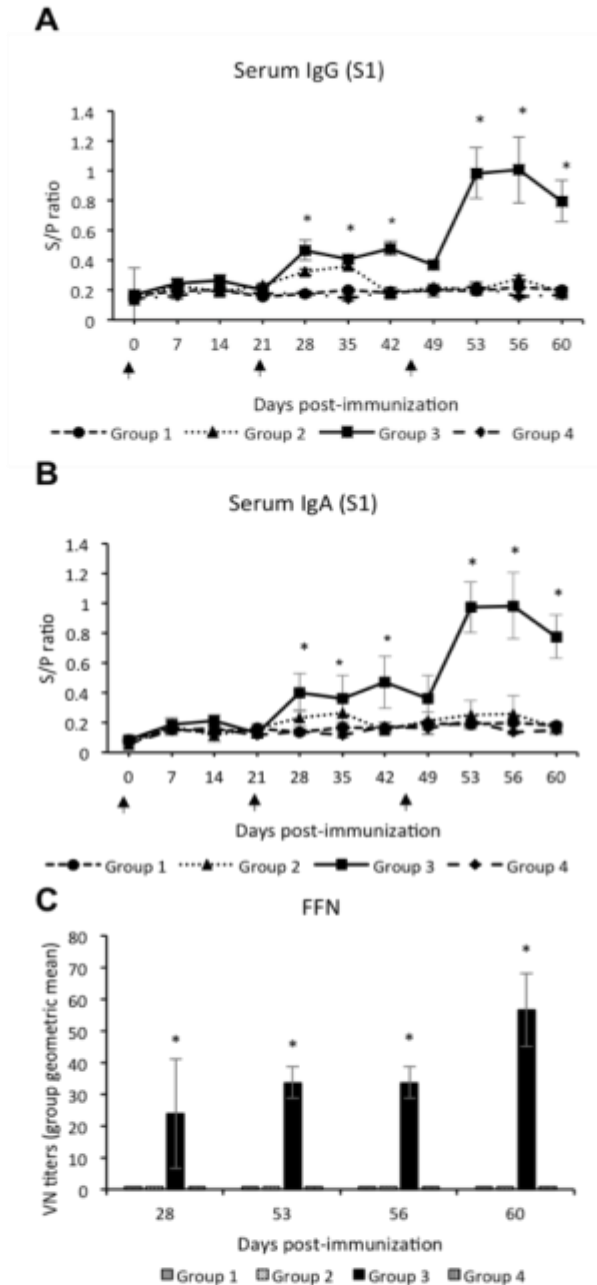


Figure 4. Immunogenicity of recombinant ORFV-PEDV-S in pigs. (A) Isotype ELISA demonstrating serum IgG antibody responses specific to the PEDV S protein. (B) Isotype ELISA demonstrating serum IgA antibody responses specific to the PEDV S protein. (C) Virus neutralizing antibody responses elicited by immunization with recombinant ORFV-PEDV-S. S/P: sample to positive ratio. Arrow heads represent immunization/booster immunizations (days 0, 21 and 45). Error bars represent standard error of the mean (SEM). Statistical significance was determined using One-way ANOVA and Tukey HSD. *, statistically significant at the 0.05 level when the mean of Group 3 was compared to the mean of Groups 1, 2 and 4.

The serological responses post-challenge with PEDV were evaluated by S1 IgG and IgA ELISAs, virus neutralization assays and N IgG ELISA. Only animals in Group 3

presented detectable anti-S and PEDV NA antibodies in serum (Fig. 5A, 5B and 5C and 6A, 6B and 6C) at the day of challenge (day 60 p.i.). Serological responses of each group were markedly different following challenge infection. Animals from Group 2 (which did not seroconvert after TC immunization) and from Group 4 (control sham-immunized group) developed lower levels of S-specific IgG and IgA and neutralizing antibodies (days 10 and 14 p.c.) when compared to animals in Group 3 (which seroconverted after IM immunization with ORFV-PEDV-S) (Fig. 6A, 6B and 6C). Notably, levels of NA antibodies detected in animals from Group 3 were 3-4 fold higher than those in animals in Groups 2 and 4 (Fig. 6C). In contrast, serum IgG responses against PEDV N were more robust in animals from Groups 2 and 4 when compared to animals from Group 3 (Fig. 6D). These differences were more pronounced at early times p.c. (day 7 p.c.; $P<0.05$), with comparable levels of anti-N antibodies being detected at later times post challenge (Fig. 6D). These results indicate an efficient priming of B-lymphocytes by IM immunization with the recombinant ORFV-PEDV-S, and further demonstrate a robust and typical secondary response, with high level antibody production following challenge with PEDV.

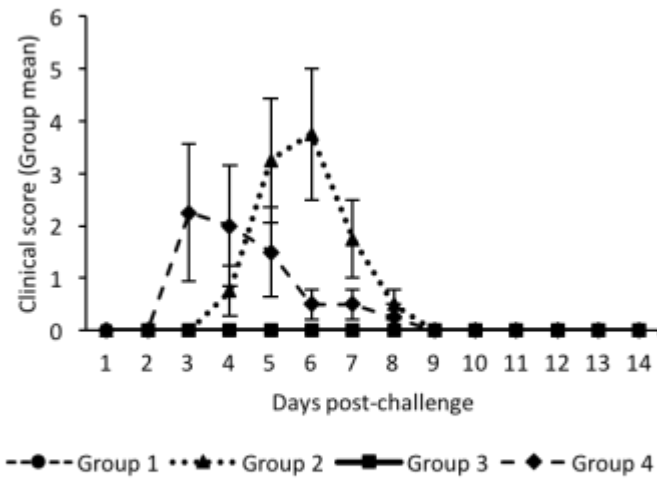
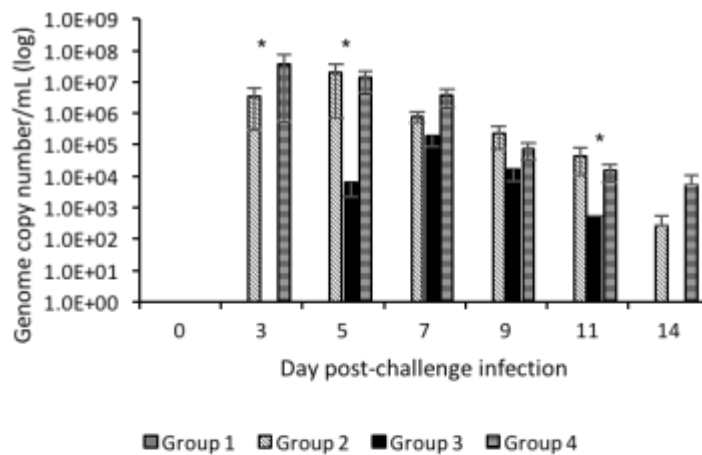
A**B**

Figure 5. Protective efficacy of ORFV-PEDV-S against PEDV challenge. (A) Average group clinical scores recorded post challenge infection with PEDV strain CO13. Clinical signs were recorded and individual daily scores assigned to all animals based on the following criteria: 0 = normal feces, 1 = pasty feces, 2 = moderate diarrhea (semi-liquid), 3 = diarrhea (liquid), 4 = severe diarrhea (very liquid), 5 = watery diarrhea (profuse diarrhea). (B) Virus shedding in feces was measured by rRT-PCR and expressed as log₁₀ genome copy numbers per mL. Error bars represent standard error of the mean (SEM). Statistical significance was determined using non-parametric Kruskal-Wallis test between groups. *, statistically significant at the 0.05 level when the mean of Group 3 was compared to the mean of Groups 2 and 4.

Table 2. Virus shedding in feces after challenge infection with PEDV.

Group	Animal ID	Virus shedding (day post-challenge) ^a						
		0	3	5	7	9	11	14 ^b
1	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
2	5	-	+	+	+	+	+	+
	6	-	+	+	+	+	+	-
	7	-	-	+	+	+	+	-
	8	-	+	+	+	+	+	-
3	9	-	+	+	+	+	+	-
	10	-	-	-	+	+	-	-
	11	-	-	-	+	+	-	-
	12	-	-	+	+	+	-	-
4	13	-	+	+	+	+	+	-
	14	-	+	+	+	+	+	-
	15	-	+	+	+	+	+	+
	16	-	+	+	+	+	+	-

^aVirus shedding was assessed by real-time PCR in fecal swabs.^bExperiment was terminated on day 14 p.c.

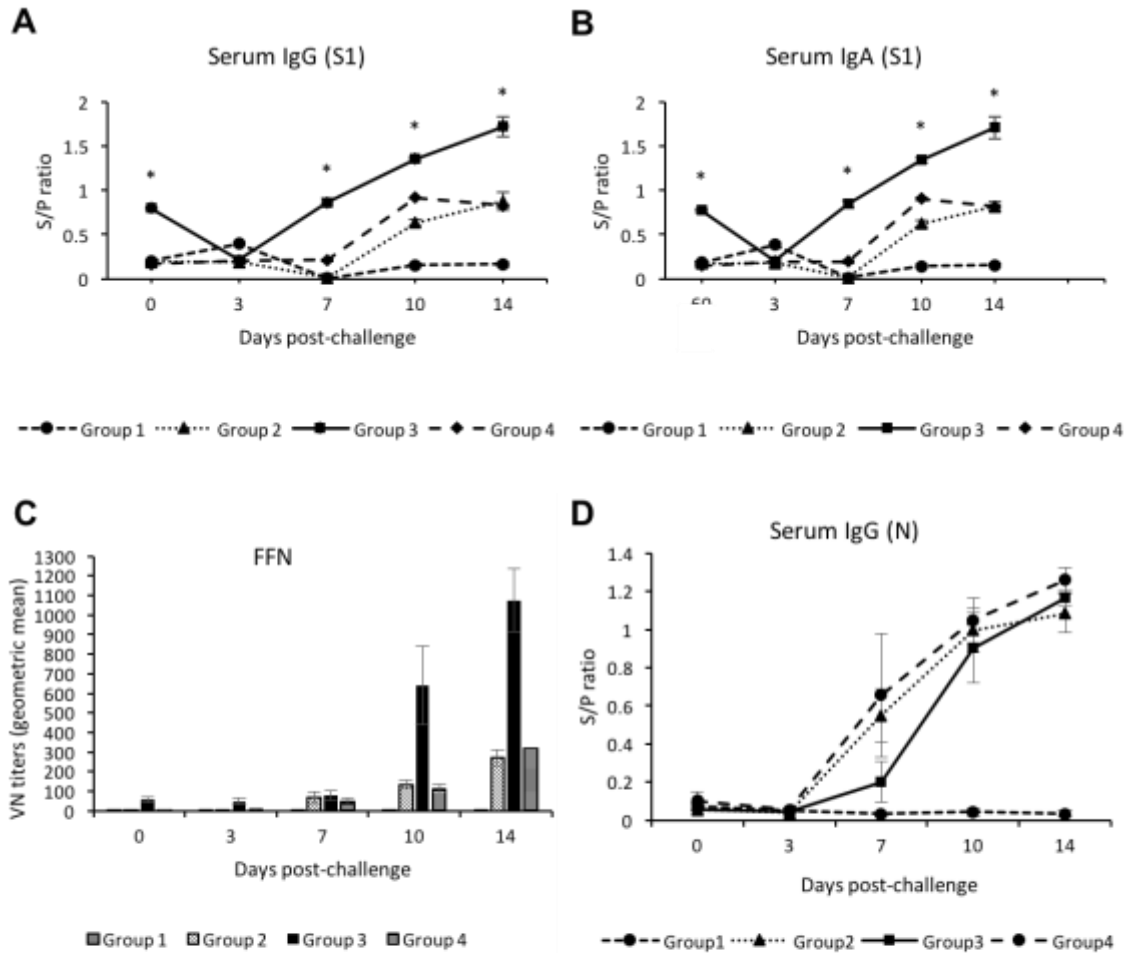


Figure 6. Serological responses following challenge infection with PEDV. (A) Isotype ELISA demonstrating serum IgG antibody responses specific to the PEDV S protein. (B) Isotype ELISA demonstrating serum IgA antibody responses specific to the PEDV S protein. (C) Virus neutralizing antibody responses. (D) Isotype ELISA demonstrating serum IgG antibody responses specific to the PEDV N protein. S/P: sample to positive ratio. Error bars represent standard error of the mean (SEM). Statistical significance was determined using One-way ANOVA and Tukey HSD. *, statistically significant at the 0.05 level when the mean of Group 3 is compared to the mean of Groups 1, 2 and 4. **, statistically significant at the 0.05 level when the mean of Group 3 was compared to the mean of Groups 2 and 4. ***, statistically significant at the 0.05 level when the mean of Group 3 was compared to the mean of Group 4.

Discussion

The immunogenicity and protective efficacy of ORFV-based vectored vaccine candidates have been demonstrated in multiple animal species^{72–77}. Notably, ORFV recombinants, based on the highly attenuated ORFV strain D1701, expressing the PRV gC- and gD- or the CSFV E2 glycoproteins have been shown to protect pigs against challenge with PRV and CSFV, respectively^{72,76,77,122}. Here we explored the potential of ORFV as a

vaccine delivery vector for enteric pathogens of swine. Using a well characterized ORFV strain (OV-IA82) ^{2,54–56} and the locus of a recently identified virulence determinant of ORFV (*ORFV121*) as insertion site, we generated a recombinant ORFV expressing the full-length PEDV S protein.

The S is the major envelope glycoprotein of PEDV and has been shown to be the main target of neutralizing antibodies ^{126–128}. Additionally, subunit PEDV vaccine candidates based on the S protein have been shown to induce protective immune responses in pigs ⁹⁰. Here, the coding sequences of the full length PEDV S were inserted into the *ORFV121* gene locus ⁵⁵ of the ORFV genome. The recombinant ORFV-PEDV-S was successfully generated (Fig. 1B, 1C and 1D), and sequencing of the virus confirmed the insertion of PEDV S- and deletion of the *ORFV121* gene from the ORFV genome. These results demonstrate the feasibility of *ORFV121* gene locus as an insertion site for heterologous antigens in ORFV-based recombinant vectors. Notably, the DNA fragment inserted into the *ORFV121* locus is ~5.2 kbp in length (full-length S- and GFP coding sequences, and promoter sequences), demonstrate that the *ORFV121* locus may accommodate large fragments of heterologous DNA. Successful expression of both PEDV S and the GFP proteins in cells infected with the recombinant ORFV-PEDV-S (Fig. 1C and 2A), confirmed the large payload capacity of the *ORFV121* gene locus and of ORFV-based vectors.

The expression and genetic stability of PEDV S carried by the recombinant ORFV-PEDV-S virus were assessed in infected cell cultures. Immunofluorescence assays performed in cell cultures infected with the recombinant ORFV-PEDV-S and permeabilized or not with Triton X-100 revealed high levels of PEDV S expression

intracellularly and on the cell surface (Fig. 2A). While intracellular expression of PEDV S may allow for antigen processing and presentation through the MHC I pathway, expression of the protein on the surface of infected cells may allow for direct recognition and uptake of the protein by antigen presenting cells (APCs) or by B lymphocytes, thus potentially leading to stimulation of both cellular and humoral immune responses ¹³⁸. An important requirement for viral vectors is the stability of heterologous genes within their genome ¹³⁹. As shown in Fig. 2B, serial passage of ORFV-PEDV-S in cell cultures *in vitro* did not affect expression of PEDV S. High levels of the PEDV S protein were detected in cell cultures infected with passages 1, 5 and 10 of the recombinant ORFV-PEDV-S, demonstrating the genetic stability of the insert into the *ORFV121* gene locus.

The immunogenicity of the recombinant ORFV-PEDV-S was evaluated in pigs following IM or TC immunizations. While IM immunization has been shown effective for other ORFV vectored antigens in pigs ^{76,77}, proof-of-concept TC immunization was used given its efficacy in inducing mucosal immune responses in other animal species ¹³⁵. Notably, animals immunized via the IM route developed robust antibody responses (IgG, IgA and NA) against PEDV, whereas no seroconversion was detected in animals immunized via the TC route (Fig. 4A, 4B and 4C). These results corroborate the findings of previous studies ^{76,77}, demonstrating that the IM route is an effective route to deliver ORFV-vectored antigens in swine. Although no neutralizing antibodies against ORFV were detected in any of the immunized animals (data not shown), it is possible that local innate/inflammatory responses elicited by skin scarification may have affected the delivery and/or expression of PEDV S by ORFV-PEDV-S in the skin, thus, precluding the

development of immune responses against PEDV S in animals immunized via the TC route.

The protective efficacy of the recombinant ORFV-PEDV-S virus was assessed in swine. Animals from Groups 2 (TC) and 3 (IM), and sham-immunized Group 4 (TC + IM), were challenged orally with a virulent PEDV strain CO13 (2×10^5 TCID₅₀). Notably, while 3 out of 4 (3/4; 75%) animals from Group 2 (which did not seroconvert after TC immunization; Fig. 4A, 4B and 4C) and 2/4 (50%) animals from control Group 4 developed characteristic signs of PED, none of the animals from Group 3 (IM, which developed serum antibody responses to PEDV) were affected. Additionally, animals from Group 3 presented reduced virus shedding in feces when compared to animals from Groups 2 and 4 (Fig. 5A and 5B; Table 2). Results from rRT-PCR performed in rectal swabs show a delayed onset and short duration shedding of PEDV by animals from Group 3 (Fig. 5B; Table 2). Together, these results demonstrate that IM immunization with the recombinant ORFV-PEDV-S protected pigs from clinical PED and reduced virus shedding following oral challenge-infection. Although results here show a strong correlation between PEDV-specific antibodies in serum, protection from clinical disease and decreased virus shedding in feces, the precise immunological mechanisms underlying these findings were not defined in our study. In future studies, it would be interesting to assess, for example, whether IM immunization with the recombinant ORFV-PEDV-S elicits secretory IgA (sIgA) responses at mucosal surfaces, or perhaps, homing of effector B or T lymphocytes to the intestinal mucosa.

Serological responses that followed challenge infection with PEDV varied significantly between immunized groups. While animals from Group 3 presented a robust

serological response, typical of a secondary immunological response, characterized by high levels of S-specific and neutralizing antibody responses to PEDV (Fig. 6A, 6B and 6C), animals from Groups 2 and 4 developed delayed antibody responses, typical of primary exposure to PEDV and characterized by lower levels of S-specific and NA antibodies (Fig. 6A, 6B and 6C). In contrast, antibody responses to N were lower in animals from Group 3 (day 7 p.c.), suggesting an early inhibition of PEDV infection/replication (Fig. 6D). Taken together, these results indicate that IM immunization with the recombinant ORFV-PEDV-S virus efficiently primed B cells, which rapidly and effectively responded upon exposure to the virus in the intestinal mucosa, leading to anamnestic antibody responses in immunized animals.

Although correlate(s) of protection for PEDV remain unknown, neutralizing secretory IgA (sIgA) antibodies are thought to play a major role in protection^{136,140–142}. sIgA seem especially important in providing lactogenic immunity and protection during the first days of life of newborn piglets^{141,143}. One of the main obstacles in eliciting lactogenic immunity to PEDV, however, is the need for local gut stimulation of IgA secreting cells (plasmablasts) and their subsequent migration to the mammary gland where they produce sIgA antibodies which are secreted in the colostrum and milk and ultimately transferred to suckling piglets (gut-mammary-sIgA axis)¹¹⁰. This has only been achieved by natural infection or by oral vaccination of pregnant sows with live PEDV vaccines^{106,137,144}. Results here show protection of nursery pigs to oral PEDV challenge after IM immunization with the recombinant ORFV-PEDV-S. Whether this vector construct is capable of eliciting lactogenic immunity and protection in neonatal piglets remains to be determined. Nevertheless, the fact that IM immunization with the recombinant ORFV-

PEDV-S led to protective immune responses in naïve animals indicates that this virus vector could be a useful tool for the control of PEDV in endemic areas. The recombinant ORFV-PEDV-S could be used to immunize naïve gilts prior to their introduction to PEDV positive farms or to boost the immunity of pregnant gilts/sows that have been naturally exposed to the virus.

In summary, here we show the successful generation of a recombinant ORFV containing the full-length S gene of PEDV into the *ORFV121* gene locus. Characterization of the recombinant ORFV-PEDV-S virus *in vitro* demonstrates efficient and stable expression of the heterologous protein in cell cultures infected with the recombinant virus. Immunization-challenge studies in pigs, show that IM delivery of the recombinant ORFV-PEDV-S elicits robust serum antibody responses in immunized animals that correlated with protection against clinical PED and decreased virus shedding in feces. These results demonstrate the potential of ORFV vectored vaccines for prevention of enteric infections in swine.

Chapter 3: Conclusion

Conclusion

ORFV-vectored vaccines have become increasingly popular as an alternative to traditional poxvirus-vectors because of their safety and ability to induce robust, dual-armed immune responses^{72,74,120,145,73,75,18,71}. Most ORFV-vectored vaccines utilize a cell-culture attenuated D1701 strain and have been used to deliver antigens from a diverse range of diseases, including the H5 protein of influenza, and the glycoproteins of rabies, pseudorabies, and classical swine fever virus^{73,75-77}. The VEGF locus has been the site of choice for DNA insertion because of its importance for ORFV virulence and pathogenesis^{72,74,120,75,77}.

In our study we explored the feasibility of ORFV121, an ORFV virulence determinant which encodes for an NF- κ B inhibitor⁵⁵, as an insertion site for heterologous genes in ORFV-based vectors. For this, the full length S protein of PEDV was used as a model antigen and a recombinant ORFV-PEDV-S was generated. Successful insertion of PEDV-S into the ORFV121 locus was confirmed by PCR (Fig. 1B), and *in vitro* expression was confirmed by IFA and Western blot (Fig. 1C and 1D).

One of the benefits of ORFV is a restricted host range that is typically limited to sheep and goats^{72,18}. Additionally, D1701 ORFV-vectors have been proven safe and highly attenuated, almost to the point of being apathogenic^{15,146}. Similarly, immunization of pigs with ORFV-PEDV-S did not result in any adverse reaction even after repeated immunization boosts. Previous studies have shown that deletion of ORFV121 significantly reduces the severity and duration of disease in the natural host of the virus⁵⁵. Experimental

infection of swine cells *in vitro* failed to result in productive virus replication (Fig. 3A and 3B).

The ORFV-PEDV-S construct efficiently expressed PEDV-S *in vitro* and *in vivo*, as evidenced by detection of the protein in infected cell cultures or detection of PEDV-S-specific antibodies in immunized animals. Animals that received IM immunizations with the ORFV-PEDV-S construct (Group 3) presented high levels of IgG, IgA, and neutralizing antibodies against PEDV (Fig. 4A, 4B, and 4C), indicating successful protein expression and delivery by ORFV-PEDV-S *in vivo*. This memory translated into complete protection of Group 3 animals from clinical PED (Fig. 5A) and reduced the magnitude and duration of virus shedding upon live virus challenge (Table 2). This protection correlated with significantly higher levels of IgG, IgA, and neutralizing antibodies in the serum of Group 3 animals when compared to those receiving TC (Group 2) or sham (Group 4) immunizations.

In conclusion, we provide further evidence of an ORFV-vectored vaccine being used to successfully protect animals against viral disease. Additionally, our study demonstrates the feasibility of ORFV121 as an insertion site for heterologous antigens in ORFV-based vectors. Although there are many other studies in which ORFV has been used to vaccinate animals experimentally, further research will be necessary to optimize ORFV-vectors to improve vaccine efficacy and promote enduring immunological memory. Determining alternate insertion sites, optimizing the immunogenicity of inserted antigens, and enhancing expression of foreign DNA may all contribute to improving the efficacy of ORFV-vectored vaccines and increasing the number of diseases that can be vaccinated against.

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